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Predictors of male microchimerism

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Abbreviations: DNA, deoxyribonucleic acid; OR, odds ratio; CI, confidence interval; CART, classification and regression tree; SAS, statistical analysis system; ICD, international classification of diseases; IQR, inter quartile range

The association between microchimerism acquired primarily through pregnancy and later disease is of increasing scientific interest. Because this line of research is new and little is known about the nature of microchimerism, studies of microchimerism are potentially vulnerable to error from confounding and reverse causation. To address the issue of confounding, we conducted an analysis of predictors of male microchimerism in 272 female participants of the Danish Diet, Cancer and Health cohort. Buffy coat DNA was tested for Y chromosome presence as a marker of male microchimerism. First, we used logistic regression and thereafter random forest modeling to evaluate the ability of a range of reproductive, lifestyle, hospital or clinic visit history, and other variables to predict whether women tested positive for male microchimerism. We found some indication that current use of contraceptive pills and hormone replacement therapy reduced the odds of testing positive for male microchimerism. However, prediction of male microchimerism presence was poor based on the available variables. Studies of the possible role of male microchimerism in maternal health and disease are therefore unlikely to be heavily confounded by the variables examined in the present investigation. More research focused on acquisition, retention and clearing of male cells in the maternal circulation is needed.

Introduction

During all pregnancies fetal cells enter the maternal circulation, where they can create a persistent microchimeric state. Based on epidemiologic observations of associations between parity and maternal health and disease as well as technical advance in detection and quantification of fetal cells, the possible roles of microchimerism are increasingly being studied and sizeable associations being reported. For example, we recently found male microchimerism presence to be associated with a 70% reduced odds of developing breast cancer, and a 4-fold increased odds of developing colon cancer.[1] Presently, little is known about acquisition, retention and clearing of male microchimerism, which is why studies on its biological roles in health and disease may be particularly vulnerable to error. First, observed associations or lack hereof may be the result of confounding from factors which are associated with both male microchimerism and the maternal outcome under study. However, because predictors of microchimerism are largely unexplored it is not straightforward to select for which factors to adjust. Next, opposite conclusions can be drawn from the same data because of reverse causation; that is the temporality of microchimerism and the outcome is rarely known. In the present manuscript we address the issue of confounding in studies of the association between male microchimerism and maternal health and disease by analyzing the predictive ability of a range of reproductive, lifestyle, hospital or clinic visit history and other variables on whether cancer-free women test positive for male microchimerism in peripheral blood.

Results

A total of 272 cancer-free women were tested for presence of male microchimerism. As recently reported, 82 (30.2%) of these tested negative and 190 (69.9%) tested positive.[1] The median number of cells tested was 107,474 (IQR 84,454–130,231) and 116,766 (IQR 94,691–142,925) in microchimerism-negative and -positive women, respectively. Among the 190 women who tested positive, the median number of male cells per 10^6 female genomes was 6.4 (IQR 2.3–18.9). Median age at enrollment was 57.2 (IQR 54.3–59.7) years in microchimerism-negative women and 56.5 (IQR 53.0–60.5) years in microchimerism-positive women, and more than three out of every four women had had one or more hospital or clinic visits prior to enrollment into the cohort (80% and 77% in microchimerism negative and positive women, respectively) (data not shown). We previously reported that approximately 4 in

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every 5 women had given birth to one or more live-born boys (82.3%).[1]

Table 1 shows the distribution and the corresponding OR (95% CI) of the variables tested for their predictive ability, according to whether cancer-free women tested negative or positive for male microchimerism. For most variables little difference was observed between strata. However, some variables were clearly unevenly distributed. For instance 55.7% of current

Table 1. Distribution and crude OR (95% CI) of male microchimerism according to reproductive, lifestyle, hospital or clinic visit history and other variables

	Male microchimerism negative (n = 82)	Male microchimerism positive (n = 190)	Crude OR (95% CI)
Reproductive variables			
Age at menarche (years)[a]			
9 to 12	19 (30.2)	44 (69.8)	0.9 (0.5–1.7)
13 to 14	36 (27.7)	94 (72.3)	1 (ref.)
≥ 15	25 (35.2)	46 (68.8)	0.7 (0.4–1.3)
Contraceptive pills			
Current	3 (50.0)	3 (50.0)	0.5 (0.1–2.7)
Former	52 (34.2)	100 (65.8)	1 (ref.)
Never	27 (23.7)	87 (76.3)	1.7 (1.0–2. 9)
Age at first pregnancy (years)[b]			
< 25	48 (29.3)	116 (70.7)	1 (ref.)
25 to 29	28 (38.9)	44 (61.1)	0.7 (0.4–1.2)
≥ 30	2 (14.3)	12 (85.7)	2.5 (0.5–11.5)
Never pregnant	4 (21.1)	15 (79.0)	1.6 (0.5–4.9)
Number of live-born boys			
0	13 (27.1)	35 (72.9)	1.2 (0.5–2.7)
1	25 (30.9)	56 (69.1)	1 (ref.)
2	23 (28.8)	57 (71.3)	1.1 (0.6–2.2)
≥ 3	21 (33.3)	42 (66.7)	0.9 (0.4–1.8)
Number of live-born girls			
0	37 (31.6)	80 (68.4)	1 (ref.)
1	29 (26.4)	81 (73.6)	1.3 (0.7–2.3)
2	14 (35.0)	26 (65.0)	0.9 (0.4–1.8)
≥ 3	2 (40.0)	3 (60.0)	0.7 (0.1–4.3)
Breast feeding (months)[c]			
0 to 2	28 (33.3)	56 (66.7)	0.9 (0.5–1.7)
3 to 5	22 (27.5)	58 (72.5)	1.2 (0.6–2.4)
≥ 6	28 (31.5)	61 (68.5)	1 (ref.)
Menopause			
Pre-menopausal	9 (25.7)	26 (74.3)	1.3 (0.6–3.0)
Post-menopausal	53 (31.4)	116 (68.6)	1 (ref.)
Unknown	20 (29.4)	48 (70.6)	1.1 (0.6–2.0)
Hormone replacement therapy			
Current	31 (44.3)	39 (55.7)	0.5 (0.3–0.9)
Former	8 (16.3)	41 (83.7)	2.0 (0.9–4.6)
Never	43 (28.1)	110 (71.9)	1 (ref.)
Lifestyle variables			
Smoking[d]			
Current	31 (35.6)	56 (64.4)	0.8 (0.5–1.5)
Former	11 (18.0)	50 (82.0)	2.1 (1.0–4.5)
Never	39 (31.7)	84 (68.3)	1 (ref.)

Table 1. Distribution and crude OR (95% CI) of male microchimerism according to reproductive, lifestyle, hospital or clinic visit history and other variables (continued)

	Male microchimerism negative (n = 82)	Male microchimerism positive (n = 190)	Crude OR (95% CI)
Alcohol (drinks/week)			
≤ 2	28 (32.2)	59 (67.8)	0.7 (0.4–1.4)
3 to 12	28 (25.9)	80 (74.1)	1 (ref.)
≥ 13	26 (33.8)	51 (66.2)	0.7 (0.4–1.3)
Physical activity (hours/week)			
0 to 9	23 (40.4)	34 (59.7)	0.6 (0.3–1.2)
10 to 19	35 (29.4)	84 (70.6)	1 (ref.)
≥ 20	24 (25.0)	72 (75.0)	1.3 (0.7–2.3)
Mediterranean diet (points)			
0 to 2	12 (36.4)	21 (63.6)	0.7 (0.3–1.5)
3 to 5	47 (28.7)	117 (71.3)	1 (ref.)
6 to 8	23 (30.7)	52 (69.3)	0.9 (0.5–1.7)
Body mass index (kg/m^2)			
< 25	36 (27.7)	94 (72.3)	1 (ref.)
25–29	31 (30.1)	72 (69.9)	0.9 (0.5–1.6)
≥ 30	15 (38.5)	24 (61.5)	0.6 (0.3–1.3)
Blood pressure			
Normal	50 (29.6)	119 (70.4)	1 (ref.)
High	32 (31.1)	71 (68.9)	0.9 (0.6–1.6)
Cholesterol[e]			
Normal	57 (29.5)	136 (70.5)	1 (ref.)
High	25 (32.5)	52 (67.5)	0.9 (0.5–1.5)
Hospital or clinic visit variables			
Infectious disease			
No	78 (29.4)	187 (70.6)	1 (ref.)
Yes	4 (57.1)	3 (42.9)	0.3 (0.1–1.4)
Malignant disease			
No	59 (28.5)	148 (71.5)	1 (ref.)
Yes	23 (35.4)	42 (64.6)	0.7 (0.4–1.3)
Blood disease			
No	78 (30.4)	179 (69.7)	1 (ref.)
Yes	4 (26.7)	11 (73.3)	1.2 (0.4–3.9)
Endocrine disease			
No	78 (30.2)	180 (69.8)	1 (ref.)
Yes	4 (28.6)	10 (71.4)	1.1 (0.3–3.6)
Mental disorder			
No	79 (29.8)	186 (70.2)	1 (ref.)
Yes	3 (42.9)	4 (57.1)	0.6 (0.1–2.6)
Nervous system disease			
No	67 (28.2)	171 (71.9)	1 (ref.)
Yes	15 (44.1)	19 (55.9)	0.5 (0.2–1.0)
Circulatory disease			
No	74 (32.2)	156 (67.8)	1 (ref.)
Yes	8 (19.1)	34 (81.0)	2.0 (0.9–4.6)

Table 1. Distribution and crude OR (95% CI) of male microchimerism according to reproductive, lifestyle, hospital or clinic visit history and other variables (continued)

	Male microchimerism negative (n = 82)	Male microchimerism positive (n = 190)	Crude OR (95% CI)
Respiratory disease			
No	75 (29.4)	180 (70.6)	1 (ref.)
Yes	7 (41.2)	10 (58.8)	0.6 (0.2–1.6)
Digestive disease			
No	73 (31.1)	162 (68.9)	1 (ref.)
Yes	9 (24.3)	28 (75.6)	1.4 (0.6–3.1)
Genitourinary disease			
No	59 (31.9)	126 (68.1)	1 (ref.)
Yes	23 (26.4)	64 (73.6)	1.3 (0.7–2.3)
Skin disease			
No	81 (31.2)	179 (68.9)	1 (ref.)
Yes	1 (8.3)	11 (91.7)	5.0 (0.6–39.2)
Musculoskeletal disease			
No	62 (28.6)	155 (71.4)	1 (ref.)
Yes	20 (36.4)	35 (63.6)	0.7 (0.4–1.3)
Injury			
No	58 (28.6)	145 (71.4)	1 (ref.)
Yes	24 (34.8)	45 (65.2)	0.8 (0.4–1.3)
Unclassified disease			
No	70 (29.9)	164 (70.1)	1 (ref.)
Yes	12 (31.6)	26 (68.4)	0.9 (0.4–1.9)
Other variables			
Age at enrollment (years)			
50 to 54	24 (24.7)	73 (75.3)	1.8 (1.0–3.4)
55 to 59	40 (37.7)	66 (62.3)	1 (ref.)
≥ 60	18 (26.1)	51 (73.9)	1.7 (0.9–3.3)
School attendance (years)			
≤ 7	27 (31.8)	58 (68.2)	1.0 (0.6–1.8)
8 to 10	42 (32.3)	88 (67.7)	1 (ref.)
≥ 11	13 (22.8)	44 (77.2)	1.6 (0.8–3.3)
Maternal brother[f]			
Yes	54 (33.5)	107 (66.5)	1 (ref.)
No	28 (26.4)	78 (73.6)	0.7 (0.4–1.2)
Maternal sister[g]			
Yes	50 (30.9)	112 (69.1)	1 (ref.)
No	32 (30.2)	74 (69.8)	1.0 (0.6–1.7)

In column two and three numbers represent counts with row percentages in parentheses. In column four numbers represent crude OR with 95% CI in parentheses. [a]Eight women had no information on age at menarche, [b]3 women had no information on age at first pregnancy, [c]19 women had no information on breast feeding, [d]1 woman had no information on smoking, [e]2 women had no information on cholesterol, [f]5 women had no information on maternal brothers and [g]4 women had no information on maternal sisters.

users of hormone replacement therapy tested positive, while the corresponding figure was 83.7% in former users and 71.9% in never-users. This corresponds to crude odds ratios of 2.0 (95% CI 0.9–4.6) among former users and 0.5 (95% CI 0.3–0.9) among current users, respectively, for testing positive for male microchimerism compared with never-users. Also, use of contraceptive pills, former smoking and age at enrollment though not statistically significant were suggestive of an association with male microchimerism presence in crude analyses. We evaluated a possible dose-response relationship between body mass index and male microchimerism positivity. In this

analysis, body mass index was entered as a continuous rather than a categorical variable in the model. The analysis, however, indicated no statistically significant relationship (data not shown).

The analysis of variable importance suggested that the most important predictors in order of magnitude were hormone replacement therapy, use of contraceptive pills, hospital or clinic visit with a nervous system disease, smoking, hospital or clinic visit with a circulatory disease, physical activity and hospital or clinic visit with a respiratory disease. However, the model-based prediction was poor. The model-based predicted probability of male microchimerism for each of the 272 cancer-free women ranged from 65% to 76%. If no information on predictor variables was available our best guess at the individual probability of male microchimerism would be 70%, which is the sample prevalence. The narrow interval of the individual random forest-based predictions suggested that the improvement in prediction conferred by the included variables was minimal. As a further illustration of the lack of predictive power of the included variables, we used the same set of variables (except body mass index) to predict obesity and found the range in predicted probabilities to be three times as wide (data not shown).

Discussion

In crude analyses we found, for example, hormone replacement therapy, contraceptive pill use and former smoking to be suggestively associated with testing positive for male microchimerism among cancer-free women. However, refined analyses addressing possible complex patterns of confounding and interaction as well as handling the limitations inferred by the small data set and lack of a priori hypotheses to be tested, suggest that none of these or any of the other variables studied are necessarily good predictors of male microchimerism in cancer-free women. Accordingly, if a similar analysis was to be conducted in a different data set, identified predictors and their order of magnitude would likely not be the same. Consequently, studies of the possible role of male microchimerism in maternal health and disease are unlikely to be heavily confounded by the variables examined in the present investigation. We encourage others to study e.g., use of hormone replacement therapy and contraceptive pills, and their association with male microchimerism before accepting these as predictors.

We assumed male microchimerism to originate from pregnancies with a male fetus. Contrary to what we expected, we found no obvious association between the number of live-born boys and detection of male microchimerism. Possible explanations include other sources of Y chromosome or male contamination. Y chromosome in women with no sons could stem from unrecognized pregnancies with a male fetus that terminated or were lost early. In support hereof Gannagé et al.[2] did not detect male microchimerism in young girls who could never have been pregnant, but found 25% of connective tissue patients who could have been pregnant, but never carried a male fetus to be positive. However, in that study none of the eight healthy control women who never carried a male fetus tested positive for male microchimerism. Also, a prior study of male microchimerism and breast cancer by Gadi et al.[3] reported 33% of healthy control women without sons to test positive. The higher frequency in our study (70%) may be the result of more unrecognized pregnancies, or the employment of a test with improved sensitivity and/or reduced specificity. A similar absence of association between parity and fetal microchimerism was also recently reported by Gammill et al.[4] However, the study by Gammill et al. also showed significantly reduced odds of maternal microchimerism with increasing parity. We have no reason to believe that samples in our study were contaminated in the laboratory because all handling of the specimens was done by female technicians.

A number of other candidate variables have been suggested as increasing the likelihood of microchimerism, most of which are pregnancy-related. Based on the notion that each new pregnancy introduces new antigens Olsen et al.[5] hypothesized that women with children fathered by different men were more likely microchimeric compared with women with the same number of children fathered by the same man. The proposed hypothesis was followed up by a study of the association between multiple fathers and subsequent maternal cancer risk,[6] but to our knowledge not verified in blood samples. Regrettably, we did not have information on fathers in our data. Based on a study by Bianchi et al.[7] showing increased microchimerism after fetomaternal hemorrhage, Khashan et al.[8] recently reasoned that caesarean section and induced abortion were good predictors of microchimerism, which in turn would affect the risk of autoimmune disorders. To our knowledge they did not verify the proposed association between mode of delivery and microchimerism in blood samples. The Danish Medical Birth Register[9] contains data on mode of delivery for all Danish births in the period from 1973 onwards. However, during this period only nine first deliveries occurred among the women in our data set and thus testing the predictive ability of mode of delivery on microchimerism positivity was not feasible. A review of the influence of pregnancy history on microchimerism presence in tissues other than blood by Khosrotehrani et al.[10] found only a history of early fetal loss to be associated with increased microchimerism presence. Sato et al.[11] reported that male microchimerism was undetectable in maternal blood 30 d or more after induced or spontaneous abortion early in pregnancy. However, in a study by Yan et al.[12] male microchimerism was frequently demonstrated in peripheral blood of healthy women who never gave birth to a son but who experienced spontaneous or induced abortion or who were nulligravid. In a recent study by Peterson et al.[13] significant fetal cell transfer was detected in maternal peripheral blood following miscarriage and termination of pregnancy, with abortions managed surgically showing 24.7 times higher detection rate compared with abortions managed medically. In our study we were unable to evaluate the influence of fetal loss on presence of microchimerism in peripheral blood because we originally excluded known miscarriages and induced abortions to reduce the risk of confounding. Y chromosome in women may also stem from a male twin, a vanished twin, or an older male sibling.[14] Twinning status of women in this study was unknown, but we found no indication of an association between having older brothers and presence of male microchimerism.

Although not yet studied, sexual intercourse without pregnancy has also been hypothesized as a source of male microchimerism.[12] Information on sexual habits was not collected in this study, which is why we could not pursue the possibility that either current or past sexual activity could predict male microchimerism positivity. Theoretically, a Y chromosome could result from meat intake, but the sequence targeted in the applied assay occurs only in human males, and we found no indication of an association between a red meat heavy diet and male microchimerism. Established non-pregnancy related factors leading to a microchimeric state include blood transfusion[15] and allo-graft reception.[16] In our study, we did not have information on transfusion and transplantation, but neither are likely sources because all women were healthy at enrollment. In future studies we aim to evaluate the influence of the above as well as other factors in the Danish National Birth Cohort.[17] Finally, the ability of a woman to integrate newly acquired haploidentical cells from sons may be influenced by her own histocompatibility genes and those of pre-existing microchimerism from her mother or other older female siblings of her son(s). Because fetal material was not available to directly genotype histocompatibility polymorphisms, future studies of microchimerism carriage rates should consider evaluation of transgenerational HLA relationships.

To our knowledge we are the first to systematically study the possible association between male microchimerism and variables related to e.g., hormone use, lifestyle factors and hospital or clinic visit history. Unlikely to be sources of microchimerism, we suggest that the possible associations are linked to retention or clearing of microchimerism obtained through pregnancy. We found current use of contraceptive pills to be suggestively associated with reduced odds of testing positive for male microchimerism. If causally linked, one obvious explanation is the inhibition of fertilization. The group of current users of contraceptive pills consisted of only six women, of whom three were positive and three were negative for male microchimerism. Besides contraception, reasons for using contraceptive pills during older reproductive age or later include control of irregular menstrual bleeding and hirsutism. Also, we detected microchimerism less often among current users of hormone replacement therapy. Because both hormone replacement therapy and contraceptive pills provide low dosages of estrogen and progestin, an alternative explanation is that these hormones somehow clear microchimeric cells. Although crudely associated, we recently reported that adjusting analyses of the association between male microchimerism and breast cancer for use of contraceptive pills and hormone replacement therapy did not alter the effect measure.[1]

A history of one or more hospital or clinic visits due to circulatory disease was crudely associated with increased odds of testing positive for male microchimerism. If truly associated, this could be due to fetal cells proliferated in situ in response to myocardial infarction as recently suggested in an editorial by Pritchard and Bianchi.[18] As additional support, epidemiological studies have demonstrated a J-shaped positive association between parity and heart disease, which is likely due to a biological effect.[19] Alternatively, male cells may stem from blood transfusions. We have no ready explanation for the possibly reduced odds of testing positive for male microchimerism in women with a hospital or clinic visit with a nervous system disease.

In assessing the role of microchimerism in women's health and disease nearly all existing studies have compared specimens collected from cases after falling ill with specimens collected from healthy controls. In such comparisons, however, one cannot separate cause from effect, which in turn may lead to opposite interpretations of the same data. For instance, less frequent detection of microchimerism in peripheral blood of women with breast cancer compared with healthy controls could suggest either a protective or a harmful effect depending on the hypothesized underlying biology. Based on the hypothesis that microchimerism increases maternal surveillance against malignant cells Gadi et al.[20] suggested a protective effect. Contrary, based on the hypothesis that microchimerism may be involved in cancer progression with selective recruitment from peripheral blood to cancer sites Dubernard et al.[21] suggested a harmful effect. We recently reported that proximity to breast cancer diagnosis did not affect the odds of testing positive for male microchimerism,[1] which supports a protective rather than a harmful effect of male microchimerism on breast cancer. Thus, in future studies of other outcomes authors should acknowledge the possibility of reverse causation and use of prospectively collected specimens when possible. All variables studied in the present manuscript concerned time prior to or time at enrollment into the cohort.

In summary, despite some indication that male microchimerism presence is predicted by e.g., current hormone use in cancer-free women, much remains in the understanding of the acquisition, retention and clearing of male microchimerism. We plan to investigate the predictive ability of a set of similar variables as well as a range of new variables in an ongoing study of the role of male microchimerism in other maternal outcomes. We encourage other researchers to do so too.

Materials and Methods

The Diet, Cancer and Health cohort. We based the present analyses on questionnaire data, anthropometric measures, and peripheral blood samples used for a recently published case-cohort study of the role of male microchimerism in developing breast and colon cancer, respectively, among women.[1] Data and blood was obtained from the population-based prospective Cancer, Diet and Health[22] cohort comprising Danish men and women enrolled during 1993–1997 when aged 50–64 y. At baseline, participants completed a detailed questionnaire on reproductive, lifestyle and other variables, and visited a study center where they had blood drawn and anthropometric measures taken. Using the unique Danish ten-digit identification number assigned to all Danish citizens, all participants were followed until 27 April, 2006 for incident cancers in the Danish Cancer Registry.[23] Migration and death data, also until 27 April 2006, was obtained for all participants from the Danish Civil Registration System.[24] Using the combined information from the Diet, Cancer and Health cohort and the linked registers, we identified a subset of participants who developed breast or colon cancer, and a subset

of controls—this latter group the subject of study here. We sampled women only because we ascertained male microchimerism by Y chromosome sequences, which should not be conventionally present in women outside of original exchange at the maternal-fetal interface. From peripheral blood buffy coat specimens from each woman, genomic DNA was isolated and tested for male microchimerism by a female technician working in a dedicated PCR-deadbox. Potential carryover contamination from prior *DYS14* amplification product was mitigated against by utilizing AmpErase TaqMan chemistry (Applied Biosystems). Using a validated TaqMan polymerase chain reaction specific to the multi-copy Y chromosome gene *DYS14*,[25] we determined male DNA presence in buffy coat DNA. Concentration of male microchimerism was estimated as number of chimeric cells per 10^6 maternal genomes. Details of the laboratory test can be found in Kamper-Jørgensen et al.[1] The study was approved by relevant scientific ethics committees in Denmark.

The Danish National Patient Register. The Danish National Patient Register[26] contains registrations on all contacts to Danish hospitals since the initiation of the register in 1977. During 1977–1994 only inpatient contacts were registered, whereas inpatient, outpatient and emergency room contacts are registered since 1995. Using the unique identification number we identified hospital or clinic visits for the identified cohort participants.

Design. As implied by the case-cohort sampling technique, cases for the original study[1] were identified after falling ill, whereas controls were identified at baseline, among all cohort members i.e., before cases fell ill. Women with known miscarriages were excluded from the study to reduce the influence of pregnancies without fetal sex identification. In the present study, we analyzed only controls. We disregard what happened after enrollment, and take interest in predictors measured before or at time of enrollment only. Thus, this is a cross-sectional evaluation of the ability of a range of reproductive, lifestyle, hospital or clinic visit history, and other variables to predict whether women tested positive for male microchimerism in peripheral blood, conducted among cancer-free women.

Analysis. Using standard logistic regression analysis, we initially modeled the crude odds ratio (OR) and associated 95% confidence interval (95% CI) of testing positive for male microchimerism in strata of each of the predictor variables. We used the stratum with the largest number of women included as reference category (OR = 1). This analysis, however, is limited by the fact that variables may confound each other's contribution or may interact in complex ways to predict male microchimerism. Also, given the limited size of the data set and the restricted prior knowledge on predictors of male microchimerism this analysis is vulnerable to poorly or over-fitted models with biased estimates of the predictive value of the variables. In order to avoid these pitfalls and robustly quantify predictors of male microchimerism, we next used the variable importance measure for conditional random forests.[27] Random forests is a widely accepted non-parametric regression method utilized in many fields, particularly for problems with a high number of variables and a low number of observations. Random forests are built from a large number of classification and regression trees (CART), which fit a model by recursive partitioning of the data set. CARTs are extremely flexible and do not make any assumptions about the functional form of the associations, which means that interactions and other nonlinear relationships do not threaten model validity. In order to "grow" a random forest, each of CARTs is fitted to a bootstrap sample from the original data. The CARTs fitted will vary between the bootstrapped samples due to random variation and because CARTs are "unstable," i.e., highly dependent of precisely from which bootstrapped sample they are estimated. It has been shown that aggregating the predictions of each of the CARTs result in predictions that are superior to those made by any single CART.[28] Variable importance is calculated as the change in prediction if the variable is left out of the random forest. This variable importance measure includes (any) main effects of the predictor variable, but also the change in prediction that arises from (potentially) complex interactions with other predictor variables.[29] Data management and logistic regression was performed in SAS 9.2 using the LOGISTIC procedure,[30] and the random forest analysis was conducted in R 2.14.1 using the library "party."[31] The random forest was built from 5,000 CARTs.

Outcome and predictors. As outcome we created a dummy variable denoting whether cancer-free women tested positive or not for male microchimerism in peripheral blood. As possible predictors we evaluated the below mentioned reproductive, lifestyle, hospital or clinic visit history, and other variables. From questionnaires completed at enrollment in the Cancer, Diet and Health cohort available information included age at menarche, use of contraceptive pills, age at first pregnancy, number of live-born boys, number of live-born girls, breast feeding, menopausal status, use of hormone replacement therapy, cigarette smoking, alcohol intake, physical activity, diet score, age at interview, duration of school attendance, number of maternal brothers and number of maternal sisters. Measurements taken at baseline at the study center included body mass index, blood pressure level and cholesterol level. Reproductive, lifestyle and other variables were categorized as shown in **Table 1**. Hospital or clinic visits prior to cohort enrollment were identified in the Danish National Patient Register and grouped by ICD-chapters. For each ICD-chapter we created a dummy variable denoting whether or not each woman had had any hospital or clinic visits according to the relevant ICD-chapter.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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